INTRODUCTION

The development of the human placenta depends on the proliferation and differentiation of trophoblastic cells. In this respect, dysfunction of trophoblasts plays an important role in the development of placental pathologies (1). Therefore, an aberrant development and differentiation of the villous syncytiotrophoblast risks the integrity of the placental barrier and causes the release of necrotic and aponecrotic trophoblast fragments (2). Histological evidences suggest a role for trophoblasts in remodeling of the uterine spiral arteries. The disruption of trophoblastic invasion and incomplete remodeling result in reduction of uteroplacental perfusion, which in turn could cause ischemia of the placenta. The alterations resulted from ischemic placenta lead to increased production of oxidative stress and stimulation of proinflammatory cytokine secretion. In this connection, it was found that production of proinflammatory cytokines such as tumor necrosis fac-
TNF-α gene expression was demonstrated at endometrial cells, decidual cells and trophoblastic cells during the trimesters of pregnancy (10). Moreover a group of pregnancy pathologies was associated with increased maternal TNF-α, which was suggested to influence fetal-maternal cross-talk during pregnancy. In this respect, because it is difficult to elucidate the role of TNF-α in such a complex process in vivo, in vitro experiments with cell lines treated with recombinant TNF-α could be illuminating. Therefore, in this research, it was aimed to examine the response of trophoblasts to TNF-α mediated cellular stress in the JAR cell line by the evaluation of proliferative, apoptotic indexes and expression levels of NF-κB which is a key signaling molecule and to assess the therapeutic potential of PTX in TNF-α induced interactions.

MATERIALS AND METHODS

Cell Culture

JAR human choriocarcinoma cell line was purchased from the American Type Culture Collection. The cells were cultured in Dulbecco’s modified Eagle’s medium/F12 medium with 10% heat inactivated fetal bovine serum under the humidified atmosphere with 5% CO₂ at 37 °C. First of all, the cells grown on coverslips were cultured with experimental doses of TNF-α (PI-RP-10921, Thermo Fisher, MA, USA) ranging from 0.1 to 1000 ng/ml; the optimal dose of TNF-α was determined in accordance with the expression levels of NF-κB. Then experimental groups were organized at 1, 6, 12 and 24 h-long incubation with blocking serum at room temperature, primary antibodies (anti-NF-κB (Sc-109, Santa Cruz, CA, USA) primary antibodies overnight at 4°C. After washing the membranes with tris-buffered saline containing 0.1% Tween 20 for 15 min, they were incubated with goat HRP-conjugated anti-rabbit secondary antibodies (PI-31460 Thermo Fisher, USA) for 1 h at room temperature. Then, following a second washing step, the membranes were also incubated with HRP-conjugated β-actin primary antibody (Sc-47778, Santa Cruz, CA, USA) as a loading control. The protein bands were visualized by using 3,3’-diaminobenzidine. Experiments were repeated three times and band intensities were quantified by densitometric analysis (Adobe Photoshop CS5) and normalized to β-actin readings.

Immunocytochemistry

JAR cells were fixed with cold methanol. Following the incubation with blocking serum at room temperature, primary antibodies against proliferating cell nuclear antigen (PCNA) (MA1-16827, Thermo Fisher, MA, USA), caspase-8 (PI-MA1-91442, Thermo Fisher, MA, USA) and NF-κB p65 (Sc-109, Santa Cruz, CA, USA) were applied overnight at 4°C. After washing with phosphate buffered saline (PBS), biotinylated secondary antibodies and horseradish peroxidase (HRP) conjugated streptavidin were applied in order. Finally, after treatment with aminoethyl carbazole, the cells were investigated with an Olympus BX-61 bright field microscope. Proliferation indexes were calculated by taking the averages of the values obtained by dividing the number of PCNA positive cells by the total number of cells in each one of the 5 different areas. The intensities of immunocytochemical stainings for caspase-8 and NF-κB were semi-quantitatively scored in accordance with the following grading system: 0 (no staining), 1+ (weak, but detectable staining), 2+ (moderate or distinct staining), and 3+ (intense staining). Experiments were repeated three times and histological scores (HSCORE) were obtained for each slide. HSCORE = Σ Pi (i + 1), where i represents the intensity score, and Pi is the corresponding percentage of the cells (11).

DAPI Staining

The cells cultured on lamellas were treated with 100 ng/ml TNF-α for 24 h and then fixed with 4% paraformaldehyde. After two times of washing with phosphate buffered saline (PBS) for 5 min, 4’,6-diamidino-2-phenylindole dihydrochloride (DAPI) stain was applied to detect apoptotic cells with their characteristic nucleus morphologies (nuclear compaction, fragmentation or semilunar appearance). The samples were investigated under an Olympus BX-61 florescence microscopy and apoptotic indexes were calculated by dividing the number of apoptotic cells by the total cell number.

Western Blot Analysis

JAR cells grown in different experimental conditions were washed with ice-cold PBS and scraped from culture flasks and then lysed with the cell lysis buffer containing a protease inhibitor cocktail to extract the total protein. The collected samples were subjected to electrophoresis on SDS-PAGE gels and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dried milk and then incubated with anti-NF-κB (Sc-109, Santa Cruz, CA, USA) primary antibodies overnight at 4°C. After washing the membranes with tris-buffered saline containing 0.1% Tween 20 for 15 min, they were incubated with goat HRP-conjugated anti-rabbit secondary antibody (PI-31460 Thermo Fisher, USA) for 1 h at room temperature. Then, following a second washing step, the membranes were also incubated with HRP-conjugated β-actin primary antibody (Sc-47778, Santa Cruz, CA, USA) as a loading control. The protein bands were visualized by using 3,3’-diaminobenzidine. Experiments were repeated three times and band intensities were quantified by densitometric analysis (Adobe Photoshop CS5) and normalized to β-actin readings.

Statistical Analyses

Statistical analyses were performed with Sigma Plot 12.0 software packages for the immunocytochemical and Western blot analyses. The data were presented as mean ± standard error (SE). Analysis between the groups were performed with One Way ANOVA test followed by Student t-test and non-parametric Kruskal Wallis-H tests for the immunocytochemistry and Western blot scores respectively. A value of p<0.05 was considered statistically significant.
RESULTS

In accordance with our preliminary studies, 100 ng/ml TNF-α was determined as an optimal dose for cellular stress induction in JAR cells. In this respect, the effects of cellular stress over the proliferation capacity of JAR cells were evaluated at the end of the 6, 12 and 24 h long incubations with TNF-α through the PCNA immunocytochemical analysis. The percentages of PCNA expressing cells after TNF-α treatment were statistically lower than the control and PCNA expression tended to increase with higher incubation time from 6th hr (Figure 1).

Second of all, cellular stress related apoptotic activity in JAR cells was investigated by evaluation of the immune reactivity of caspase-8 at the end of the 1, 6, 12 and 24 h of treatment with TNF-α. The caspase-8 immune reactivities of all the TNF-α applied groups were significantly higher than the control group and 1 h of TNF-α treatment brought about the highest caspase-8 immune reactivity (Figure 2A). Furthermore, cells treated with TNF-α for 24 h were compared with the non-treated ones by DAPI staining which is specific for DNA in order to show apoptosis related morphologic changes (Figure 2B-D). The number of cells having characteristic apoptotic nuclear morphology in the TNF-α treated group was significantly higher than the ones in the control (Figure 2B).

NF-κB signaling is one of the key pathways for the regulation of expressions of genes related to cell survival and proliferation, and activated by TNF-α (5,6). Therefore, nuclear NF-κB expression levels of the JAR cells were evaluated at the end of the 1-, 6-, 12- and 24 h long incubation with TNF-α through the immunocytochemical (Figure 3A-E and Table 1) and Western blot analysis (Figure 3F). It was found that 1 h long induction with TNF-α was effective to induce NF-κB expression significantly (Table 1). At this point, we assessed the effects of dif-

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Figure 1. Proliferation indexes expressed with percentages (%) of PCNA positive cells for the control and TNF-α treated cell groups with different time intervals. \( ^a P < 0.05 \) vs 12- and 24-hour control groups. \( ^b P < 0.05 \) vs control groups. \( ^c P < 0.05 \) vs 12- and 24-hour of TNF-α treated cell groups.

Figure 2. Caspase-8 immune reactivities of control and TNF-α treated cell groups with different time intervals (A). Apoptotic cell rates for the control and 24-hour TNF-α treated cell groups (B). Representative photomicrographs of control (C) and 24-hour long TNF-α treated cells (D) (arrow: normal nucleus morphology; star: apoptotic nucleus morphology, 100X). \( ^a P < 0.05 \) vs TNF-α treated cell groups. \( ^b P < 0.05 \) vs 6-, 12- and 24-hour of TNF-α treated cell groups. \( ^c P < 0.05 \) vs 12- and 24-hour of TNF-α treated cell groups. \( ^d P < 0.05 \) vs control group.
different doses of PTX, which has been reported as an inhibitory molecule for TNF-α on NF-κB expression levels. NF-κB immunoreactivities disappeared in all of the nuclei of cells treated with only 1 mM, 10 mM and 20 mM PTX. However, it was found that only 10 mM PTX was able to completely abolish the basal level of nuclear NF-κB expression, and significantly reduced the nuclear NF-κB expression induced by 1-h long incubation with 100 ng/ml TNF-α (Figure 4, Table 2).

Table 1. H scores for nuclear expressions of NF-κB average±SD in the control and TNF-α groups with different time intervals

<table>
<thead>
<tr>
<th>Groups</th>
<th>Average±SD</th>
</tr>
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<tbody>
<tr>
<td>Ctrl</td>
<td>169±7</td>
</tr>
<tr>
<td>TNF-α (1h)</td>
<td>329±7&lt;sup&gt;a&lt;/sup&gt; &lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>TNF-α (6h)</td>
<td>219±9&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>TNF-α (12h)</td>
<td>302±11&lt;sup&gt;a&lt;/sup&gt; &lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>TNF-α (24h)</td>
<td>224±7&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
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SD: standard deviation, <sup>a</sup>P<0.05 vs. control (ctrl) group, <sup>b</sup>P<0.05 vs. 6 h and 24 h long TNF-α treated groups.

Table 2. H scores for nuclear expressions of NF-κB average±SD in the control, TNF-α (1h) and PTX groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Average±SD</th>
</tr>
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<tbody>
<tr>
<td>Ctrl</td>
<td>169±7</td>
</tr>
<tr>
<td>TNF-α (1h)</td>
<td>329±7</td>
</tr>
<tr>
<td>10 mM PTX</td>
<td>0</td>
</tr>
<tr>
<td>10 mM PTX + TNF-α (1h)</td>
<td>180±10&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
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SD: standard deviation, <sup>a</sup>P<0.05 vs. TNF-α (1h) treated group.
Furthermore, Western blot analysis showed that total NF-κB expression was slightly increased with the TNF-α treatment and PTX application reduced total NF-κB expressions significantly compared to the control and only TNF-α treated ones (Figure 5).

**DISCUSSION**

Invasiveness of syncytiotrophoblasts is required for remodeling of spiral arteries during pregnancy. Inadequate invasion of trophoblasts leads to a deficient rupture of spiral arteries in the muscular layer, which in turn leads to disruption of utero-placental circulation (12,13). Interrupted arterial blood supply results in an increased generation of inflammatory cytokines like TNF-α (14,15). In this respect, determination of increased level of TNF-α in the plasma of preeclamptic pregnant women has shown that TNF-α could be used as an inducing agent for the establishment of experimental models (16). TNF-α takes part in induction of rapid transcription of genes associated with the regulation of proliferation, cell survival, inflammation and differentiation mainly through activation of the NF-κB pathway (6). For those reasons, it was aimed to evaluate the effects of TNF-α as a potential cellular inducer on JAR trophoblastic cells in our research. First of all, the proliferation indexes of JAR cells were evaluated and it was found that following 6 h of incubation with TNF-α, proliferating cell numbers was significantly reduced which could be interpreted as TNF-α having an anti-proliferative effect on trophoblastic cells. Caspase-8 is an initiator caspase, and is predominantly located in mitochondria as a pro-enzyme and released upon apoptotic stimulation like TNF-α (17). Caspase-8 is a prototypic caspase of apoptotic death receptor pathways and activates its ligand by binding to members of the TNF-α receptor superfamily (18,19). In this respect, at the end of the 1 h, cells treated with 100 ng/ml TNF-α had significantly higher caspase-8 immune reactivity. As an initiator caspase, caspase-8 plays an important role in TNF-α induced cellular stress, which could lead to apoptosis in the end. In this respect, morphologic changes like nuclear fragmentation are a late stage event for apoptosis (20). Therefore, the long-term effects have been showed by DAPI staining at the end of the 24 h long incubation with TNF-α. Those results support that TNF-α could be one of the main cytokine factors taking part in the placental pathologies, which are associated with trophoblast apoptosis such as preeclampsia. In addition, 100 nm/ml TNF-α treatment is quite effective for setting up an in vitro preeclamptic experimental model in trophoblastic JAR cells. Therefore, we wanted to know the role of the NF-κB signaling pathway in those processes. In the absence of inducing stimuli, NF-κB molecules are in an inactive state in the cytoplasm. Upon activation by TNF-α, NF-κB is transferred to the nucleus, and regulates the associated genes (21,22). In this respect, the effects of TNF-α treatment on the nuclear NF-κB expression levels in different time intervals were evaluated. In our experiments, we showed that a 1 h long induction with TNF-α brought about a significant increase in the total and nuclear expression of NF-κB. Even though the nuclear expression of NF-κB was fluctuating with increasing time of TNF-α stimulation, the decrease in proliferative capacity and increase in apoptotic activity are quite consistent with longer incubation time. Similarly, increased NF-κB expression induced with TNF-α was demonstrated in ED27 cells, which are immortalized trophoblast-like cells (23). Furthermore, increased NF-κB expression was also shown for syncytiotrophoblastic cells of preeclamptic placental tissues (24). As a result, we could define a positive feedback loop between TNF-α and NF-κB expressions in JAR trophoblastic cells which possibly participates in progressive aggravation of inflammation in the placenta.

PTX, which is a methylxanthine derivative and a non-specific inhibitor of cAMP phosphodiesterase, is generally applied as a pharmacologic agent for improvement of circulation in peripheral vascular disorders (8,25). Moreover, possible therapeutic effects of PTX as an inhibitor of TNF-α synthesis have been investigated in various diseases (26,27). Importantly, it was noted that PTX treatment has a reducing effect on plasma levels of proinflammatory cytokines including TNF in addition to its antioxidant effects (9). In our study, we investigated the optimal dose of PTX for inhibition of TNF-α induced NF-κB expression and it was found that 10 mM PTX application was quite effective in the reduction of TNF-α stimulated nuclear expression of NF-κB. It was reported that NF-κB translocation to nuclei was blocked by PTX application in TNF-α stimulated vascular smooth muscle cells (28). Therefore, PTX could be a potential therapeutic agent for the treatment of TNF-α related placental pathologies.

**CONCLUSION**

In summary, the present study showed that incubation with TNF-α leads to a decrease in proliferation capacity and an increase in apoptotic activity and NF-κB signaling in JAR syncytiotrophoblastic cell lines. In addition to all of this, PTX could be a potential regulatory agent for TNF-α synthesis have been investigated in various diseases (26,27). Importantly, it was noted that PTX treatment has a reducing effect on plasma levels of proinflammatory cytokines including TNF in addition to its antioxidant effects (9). In our study, we investigated the optimal dose of PTX for inhibition of TNF-α induced NF-κB expression and it was found that 10 mM PTX application was quite effective in the reduction of TNF-α stimulated nuclear expression of NF-κB. It was reported that NF-κB translocation to nuclei was blocked by PTX application in TNF-α stimulated vascular smooth muscle cells (28). Therefore, PTX could be a potential therapeutic agent for the treatment of TNF-α related placental pathologies.
REFERENCES


